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Cytokines and Cytotoxic Pathways in Engraftment Resistance to Purified Allogeneic Hematopoietic Stem Cells

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ABSTRACT

The way that allogeneic hematopoietic cells are rejected is not completely understood. Regimen-resistant populations, including natural killer (NK) cells and lymphocytes, are thought to mediate the allograft barrier. In this report, the mechanism by which recipient cell populations resist engraftment of purified allogeneic hematopoietic stem cells (HSCs) was examined in mice. To define the immunoregulatory pathways involved in allogeneic hematopoietic cell resistance, HSC transplantations were performed in immune-defective recipients. Recipients were wild-type mice treated with α-NK cell antibodies or knockout strain mice lacking expression of CD8, perforin, Fas ligand, or 1 of the following cytokines: tumor necrosis factor α, transforming growth factor β, interferon γ, interleukin 4, or interleukin 10. Elimination of a single cytotoxic pathway was ineffective in reducing engraftment resistance, although mice treated with a polyclonal antibody that recognizes NK-cell determinants or CD8 expression showed a profound reduction in the engraftment barrier. Posttransplantation chimerism analysis revealed regeneration of host hematopoiesis in some experimental groups. These studies show, for the first time, that elimination of selected cytokines does not alter allogeneic hematopoietic resistance. Furthermore, the chimerism data reinforce the importance of competition for HSC niches in conjunction with immune mechanisms in resistance to long-term HSC engraftment.

KEY WORDS

Cytokines • Perforin • Fas ligand • Stem cell transplantation • Engraftment

INTRODUCTION

In allogeneic hematopoietic cell transplantation (AHCT) for the treatment of hematologic malignancies or bone marrow (BM) failure states, the benefits conferred by the hematopoietic graft are to both replace the diseased cells in the recipient and mediate graft-versus-tumor effects [1,2]. Unfortunately, conventional hematopoietic grafts can also induce graft-versus-host disease (GVHD), which remains an important cause of morbidity and mortality after AHCT [3,4]. GVHD occurs when mature immune cells, primarily T lymphocytes, contained within a hematopoietic graft recognize and direct responses against healthy host tissues. Previous attempts to eliminate graft T cells resulted in reduced GVHD; however, significant increases in graft failure were also observed [5-9]. Graft failure that occurs in recipients conditioned for transplantation with myeloablative regimens is invariably fatal [6,10,11]. Thus, most transplant centers continue to use unmanipulated BM or mobilized peripheral blood despite the risk of GVHD, and resistance to engraftment remains a major obstacle to the future use of engineered hematopoietic grafts composed of purified populations.

The way that allogeneic hematopoietic grafts are recognized and eliminated by recipients is not completely understood. The studies of Cudkowicz and Bennett [12] and Kiessling et al. [13] and later of Bennett [14] showed that resistance to hematopoietic cells involves biological processes distinct from solid tissue rejection. Differences in the genetics and the

cell populations that mediate resistance have been described [14]. Studies in mice show that whereas solid organs transplanted from inbred strain parents into F(1) offspring uniformly result in graft acceptance, this genetic pattern of acceptance is not always observed for hematopoietic grafts, because F(1) offspring can demonstrate vigorous resistance—a phenomenon initially termed hybrid histocompatibility (Hh) resistance [14]. Hh was later renamed to hemopoietic histocompatibility 1 (a more broadly applicable term) when similar major histocompatibility complex (MHC)–linked patterns of resistance were noted to apply to BM allografts. Hh resistance occurs despite conditioning of recipients with lethal doses of radiation (XRT), which is a treatment that results in nearly complete elimination of host hematopoietic cells, including stem and progenitor cells and T and B lymphocytes. It is known that natural killer (NK) cells play a central role in Hh resistance [15-17]. However, NK cells do not participate significantly in solid organ transplant rejection [18,19].

Because NK cells kill susceptible targets, it has been proposed that the cytotoxic functions of NK cells mediate hematopoietic cell resistance [19-21]. Three principal cytotoxic pathways can be used. One depends on exocytosis of granules containing perforin and granzymes [22-26], the second on Fas (Apo-1; CD95) and its ligand (FasL) [27-30], and the third on secreted molecules [31-34]. Prior reports showed that in animals lacking intact pathways of granule and/or Fas killing, resistance to hematopoietic cell engraftment was unimpaired [35-39]. Thus, it was suggested that certain lymphokines secreted by NK cells with known cytotoxic or growth-inhibitory effects are the primary effectors of hematopoietic cell resistance.

In this report, the mechanisms by which NK and other recipient cell populations resist engraftment of purified hematopoietic stem cells (HSCs) were examined in mice. Analogous to the clinical experience with T cell–depleted BM, HSCs are more vigorously resisted as compared with unfractionated BM [40]. Here, purified allogeneic HSCs devoid of facilitating cells that could confound the analysis were transplanted across a highly resistant mouse strain combination into wild-type mice or mice with genetic defects in expression of specific secreted molecules, including tumor necrosis factor (TNF)–α, transforming growth factor (TGF)–β, and polarizing T-helper (Th)1 and Th2 cytokines. perforin or FasL expression were also used as recipients. We observed that elimination of any 1 cytotoxic pathway tested here did not reduce the barrier to allogeneic HSC engraftment. In some recipient groups reconstitution of host hematopoiesis was noted, suggesting that the HSC barrier is a composite of both immune-mediated resistance and competition for stem cell niches.

MATERIALS AND METHODS

Mice

Animal care was provided in accordance with the procedures outlined in the *Guide for the Care and Use of Laboratory Animals* (NIH Publication 86-23; 1985). HSC donors were 5- to 7-week-old AKR/J ($H2^k$) strain mice. Recipient mice were 8- to 16-week-old wild-type C57BL/6 (B6.WT, $H2^b$) or genetically engineered knockout mice for the following genes on the C57BL/6 background: B6.IL-$4^{-/-}$ (B6.129P2-$Il4^{tm1Cgn}$), B6.IL-$10^{-/-}$ (B6.129P2-$Il10^{tm1Cgn}$), B6.TNF-$\alpha^{-/-}$ (B6.129-$Tnfrsf1a^{tm1Mak}$), B6.TGF-$\beta^{-/-}$ (B6.129S2-$Tgfb1^{tm1Doe}$), B6.IFN-$\gamma^{-/-}$ (B6.129S7-$Ifng^{tm1Ts}$), B6.$pfp^{-/-}$ (C57BL/6-pfp^{tm1Sdz}), and B6.gld (B6.Smn.C3H-$Fasl^{gld}$). Recipients were purchased from Jackson Laboratory (Bar Harbor, ME), and founder mice were then bred and maintained in the Stanford University Department of Laboratory Animal Medicine under pathogen-free conditions by using filter-top cages and temperature-controlled and light-cycled rooms.

HSC Purification

Whole BM cells were obtained from donors by flushing tibias and femurs with Hanks balanced salt solution (Applied Scientific, San Francisco, CA) supplemented with 2% fetal calf serum (Gibco BRL, Carlsbad, CA) by using a 25-gauge needle and syringe. HSCs were isolated by a modification of the method described by Spangrude et al. [41]. In brief, whole BM cells were enriched for *c-Kit* (CD 117, stem cell factor receptor) by staining with a biotinylated *c-Kit* monoclonal antibody (mAb) and run through a magnetically activated cell-sorting streptavidin-conjugated magnetic bead column (Miltenyi Biotec, Auburn, CA). The *c-Kit*–enriched fraction was stained with fluorescein isothiocyanate (FITC)–conjugated α–Thy-1.1 (19×E5), Texas red–conjugated α–Sca-1 (E13-161), allophycocyanin-conjugated α–*c-Kit* (2B8), and a mixture of phycoerythrin-conjugated lineage-specific mAbs, as follows: α-B220 (6B2), α-CD3 (145-2C11), α-CD5 (53-7.8), α-CD4 (GK-1.5), α-CD8 (53-6.7), α–Gr-1 (8C5), α–Mac-1 (M1/70), and α-TER119 (TER199). The conjugated mAbs were the gift of Dr. I. Weissman, of the Department of Pathology, Stanford University, except for α-CD3 mAb (145-2C11) and α-CD8 mAb (53-6.7), which were obtained from Pharmingen (San Diego, CA). Propidium iodide stain at 1 mg/mL was used to exclude dead cells. Cells were sorted on a dual-laser Vantage sorter (Becton Dickinson, Mountain View, CA) made available through the fluorescence-activated cell sorting (FACS) shared user group at Stanford University. Verification of sorted cells was obtained by ungated reanalysis. After sorting for $FITC^{lo}$, Texas red^{hi}, $allophycocyanin^{hi}$, and $phycoerythrin^{-/lo}$, the *c-Kit*$^+$Thy-1^{lo}Lin$^{-/lo}$Sca$^+$ (KTLS

HSCs) cells were checked by FACS reanalysis and determined to be >99% pure.

Recipient Preparation and HSC Transplantation

Recipient mice were conditioned for transplantation with lethal XRT on day 0 relative to transplantation at a total dose of 950 cGy. A Phillips unit irradiator (250 kV; 15 mA) was used to deliver the XRT in 2 divided (475 cGy each) doses 3 to 4 hours apart. After XRT, mice were maintained on antibiotic water containing 10^6 U/L of polymyxin B sulfate and 1 g/L neomycin sulfate. Antibody-treated mice received α-ASGM1 (WAKO Chemicals, Dallas, TX) at 50 μg intravenously on day −7 and by intraperitoneal injection on day −1. Purified HSCs were injected on day 0 in titrated numbers (300, 1000, 3000, and 6000) in a volume of 100 μL per mouse. Cells were injected into the suborbital venous plexus of recipient mice. The biological activity of the HSC transplant was reconfirmed by injection of 100 or 200 cells into lethally irradiated congenic recipients (AKR/Cu; $H2^k$), which were killed for day −12 spleen colony-formation unit assay analysis (data not shown).

Detection of Hematopoietic Chimerism

Chimerism was assessed at 6 weeks after transplantation by FACS analysis of erythrocyte-depleted peripheral blood with donor-specific staining for the MHC class I $H2^k$ markers versus B cells (α-B220), granulocytes (α–Gr-1), macrophages/monocytes (α–Mac-1), T cells (α-CD3), and NK cells (α-DX5); all antibodies were obtained from Pharmingen. FACS analysis was performed by a modified FACS II system (Becton Dickinson) equipped with logarithmic amplifiers. Data are presented as contour plots on log-10 scales of increasing green and red fluorescence intensity.

Subset Staining Analysis

Splenocytes, peripheral blood, and BM cells from mice in selected treatment groups were analyzed by a 3-step FACS staining for α-ASGM1 versus the following lineage subset markers: CD3, CD4, CD8, Gr-1, NK1.1, and Mac-1. Unconjugated α-ASGM1 antibody was used as the first step, followed by 2 washes in media. A second step, FITC-labeled goat anti-rabbit mAb (GAR-FITC), was then added. The lineage subset mAbs (directly conjugated to phycoerythrin) were then added as the third staining step. Staining with GAR-FITC mAb alone was used to rule out nonspecific binding.

Statistical Analysis

For each HSC dose, the 100-day survival experience was compared between the control and each treatment group. The log-rank test was used, and the nominal *P* values are reported. When separate experiments were performed, the *P* values reflect the results of each individual experiment.

RESULTS

Quantitation of the Barrier to Allogeneic HSC Engraftment

In a prior report we demonstrated in several allogeneic mouse strain combinations that the relative resistance to engraftment of purified HSCs can be measured by the numbers of HSCs needed to rescue lethally irradiated recipients [40]. HSC transplantation in syngeneic or CD45 congenic mice requires only 100 to 200 HSCs for rescue [41], and, in general, as the genetic disparity increases between donor and recipient, more HSCs are required. In this study, an MHC-mismatched strain combination of AKR/J ($H2^k$) into B6.WT ($H2^b$) mice was selected so that the many immune-defective mice that exist on the C57BL/6 background could be used as recipients. Because resistance to long-term engraftment of KTLS HSC was not previously characterized in this strain combination, rescue of irradiated B6.WT mice was tested by infusion of titrated numbers of AKR/J HSCs. Recipients were followed up for >90-day survival and evaluated for peripheral blood chimerism. Survival data combined from 2 separate experiments are shown in Figure 1A. A total of 6000 AKR/J HSCs rescued 70% of B6.WT mice from aplasia, whereas 3000 HSCs rescued only 10%, which correlated with a relatively high level of resistance as compared with another MHC-mismatched strain combination (B6.WT [$H2^b$] into BALB/c [$H2^d$]). In B6.WT into BALB/c mice, we previously noted that 6000 HSCs rescued all recipients and that 3000 HSCs rescued >60% [40]. The blood of surviving AKR/J into B6.WT recipients was analyzed by FACS analysis at 6 weeks after transplantation. Figure 1B is a representative profile of the peripheral blood chimerism of 1 of these B6.WT recipients. Similar to our observations in other mouse strain combinations [40,42], lethally irradiated, HSC-engrafted mice demonstrated significant levels of radioresistant host T cells, whereas the other white blood cell lineages were nearly all donor derived (>95% donor type).

Elimination of NK and/or $CD8^+$ Cells Reduces Resistance to HSCs

Cells with NK markers and $CD8^+$ cells have been shown to mediate resistance to engraftment of BM in different mouse strain combinations [19]. To determine whether the activities of these cell populations are similarly relevant to the engraftment barrier of AKR/J HSCs into B6.WT mice, B6.WT recipients were treated with a polyclonal antibody, α-ASGM1,

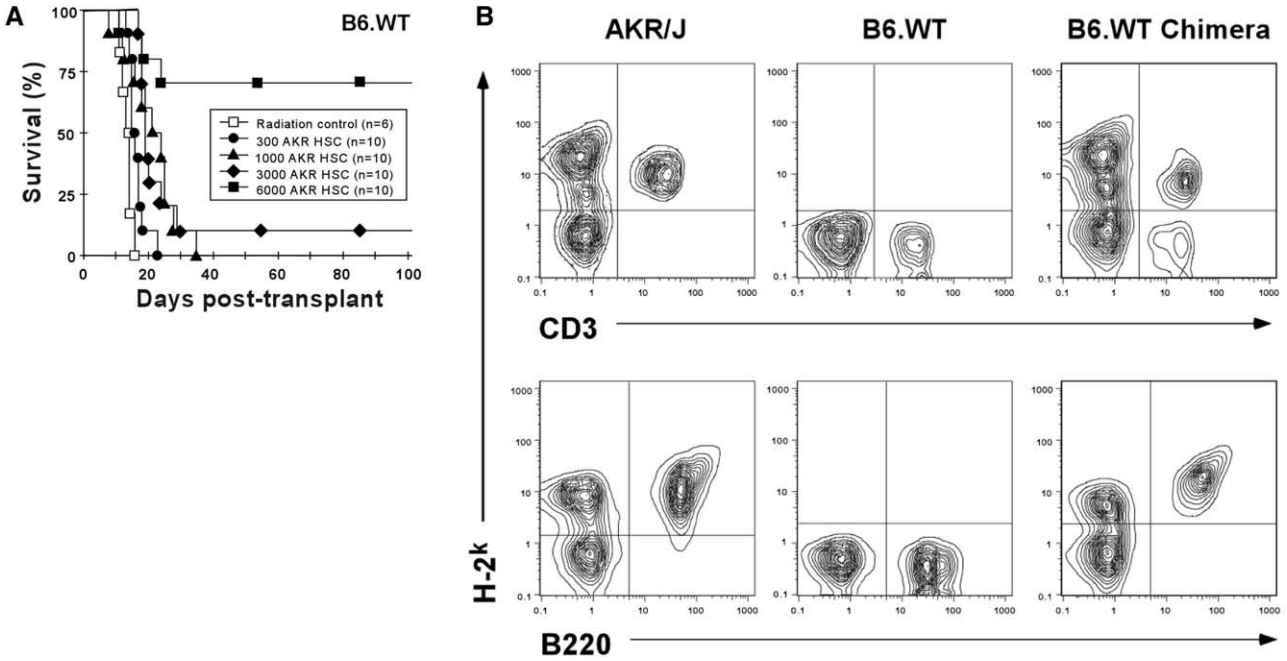

Figure 1. Survival and chimerism analysis of lethally irradiated mice that underwent transplantation across MHC barriers. Titrated doses of MHC-mismatched AKR/J ($H2^k$) HSCs were injected into lethally irradiated B6.WT ($H2^b$) mice. A, Survival of mice that underwent transplantation with 300, 1000, 3000, or 6000 HSCs was compared with that of XRT controls (950 cGy of radiation). Results are pooled from 2 independent experiments. B, Flow cytometric analysis of peripheral blood from B6.WT mice performed 6 weeks after they received 6000 HSCs. Detection of hematopoietic chimerism was performed with donor-specific staining for the class I H2 marker (α-$H2^k$) versus cell lineage markers. Shown are profiles of B- and T-cell chimerism from the peripheral blood of a representative engrafted B6.WT recipient; these are compared with blood profiles from control unmanipulated B6.WT and AKR/J mice. Note that the engrafted mouse is a partial T-cell chimera, whereas the B cells are all donor derived.

known to abolish both NK cell and cytotoxic T lymphocyte (CTL) activity in vivo [43,44]. Figure 2A shows that the addition of α-ASGM1 resulted in significantly improved survival as compared with XRT only. A total of 6000 and 3000 AKR/J HSCs rescued 100% of recipients (P = .317 and P = .013, respectively), 1000 HSCs rescued 80% of recipients (P = .007), and 300 HSCs rescued 60% of recipients (P=0.037). Blood chimerism studies at 6 weeks after transplantation revealed that most mice that underwent transplantation with >1000 HSCs were converted to nearly complete donor type. It is interesting

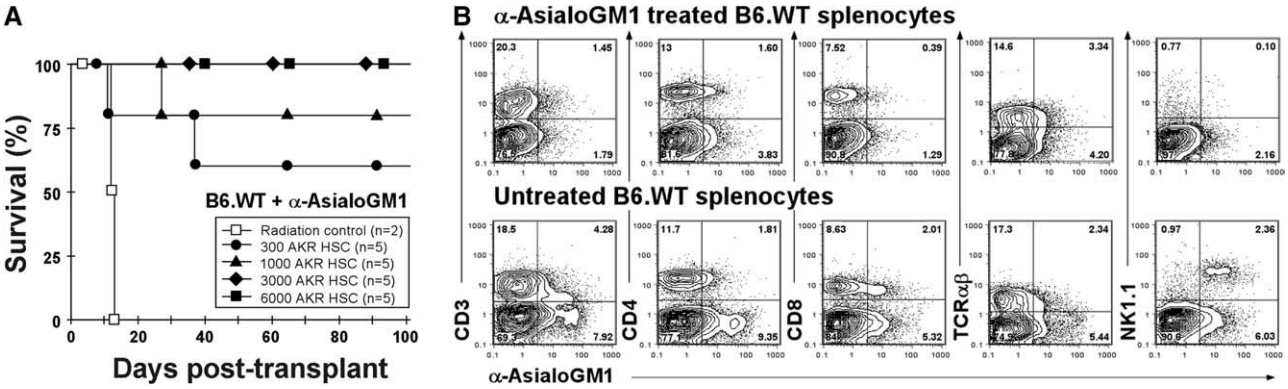

Figure 2. Effects of α-ASGM1 polyclonal antibody pretreatment on the survival of lethally irradiated mice that underwent transplantation across MHC barriers. Lethally irradiated B6.WT mice received antibody treatment on day −7 (intravenously) and on day −1 (intraperitoneally) before transplantation of titrated doses of MHC-mismatched AKR/J HSCs. A, Survival of mice that underwent transplantation with 300, 1000, 3000, or 6000 HSCs is compared with that of XRT controls (950 cGy of radiation). B, Flow cytometric analysis of spleen cells 7 days after intravenous and intraperitoneal administration of 50 μg of α-ASGM1 antibody of treated (top) and untreated (bottom) B6.WT mice. Detection of ASGM1-expressing T-cell subsets (CD3, CD4, CD8, and T-cell receptor αβ) and NK cells (NK1.1) was performed by FACS analysis by using α-ASGM1 as a staining reagent. Note the depletion of lymphocyte subsets in the α-ASGM1 antibody–treated mouse.

Table 1. *Survival and Chimerism Analysis after Transplantation of Purified HSCs from AKR/J into B6.WT and α–ASGM1–Treated Recipients*

Recipient	% Survival/% Donor Chimerism for HSC Dose (No. Mice Analyzed)			
	300 KTLS	1000 KTLS	3000 KTLS	6000 KTLS
B6.WT	0/0 (10)	0/0 (10)	10/100 (10)	70/100 (10)
α-ASGM1–treated B6.WT	60/0 (5)	80/75 (5)	100/100 (5)	100/100 (5)

Shown are the percentage of recipients that survived for a given HSC dose and the percentage of these survivors that were chimeric. Recipients were prepared for transplantation with lethal XRT with or without treatment with α-ASGM1. Flow cytometric analysis was performed for detection of chimerism with donor-specific staining for class I $H2^k$ marker versus B cells (α-B220), granulocytes (α–Gr-1), macrophages/monocytes (α–Mac-1), T cells (α–CD3), and NK cells (α–DX5) of surviving mice 6 weeks after HSC transplantation against MHC barriers. Mice deemed chimeric had on average > 60% donor-derived blood T cells. Non–T-cell chimerism was best quantitated by α-B220 staining, which was, on average, > 95% donor type. Nonchimeric recipients were < 5% donor type in all lineages. Data are pooled from 2 independent experiments.

KTLS indicates *c-Kit*$^+$Thy-1^{lo}Lin$^{-/lo}$Sca-1$^+$ HSCs.

to note that despite a 60% survival rate in the group of mice that underwent transplantation with 300 HSCs, none of the mice was a donor chimera (Table 1). Thus, in this resistant strain combination, targeting radioresistant ASGM1$^+$ host populations significantly decreased the barrier to HSC engraftment. Furthermore, at the lower HSC dose, it seemed that although antibody pretreatment allowed donor elements to rescue mice from aplasia, host hematopoiesis was responsible for long-term recovery.

Identification of α-ASGM1 Targets

To identify by phenotype the ASGM1$^+$ cells in the recipients targeted by the antibody, α-ASGM1 was used as a staining reagent in FACS analyses. Double staining of ASGM1$^+$ cells was performed by using CD3, CD4, CD8, T-cell receptor αβ, NK1.1, and Mac-1 mAbs. Hematopoietic cell populations from B6.WT mice were analyzed and compared with B6.WT mice treated with α-ASGM1 only, XRT only, or XRT plus α-ASGM1. Figure 2B shows the FACS profiles of splenocytes from a representative wild-type mouse versus a mouse treated 7 days previously with α-ASGM1 only. As expected, the predominant population bound by α-ASGM1 double-stained for the NK1.1 marker. In addition, the α-ASGM1 antibody also labeled subsets of other populations, including CD3, CD4, CD8, and Mac-1. Proof that the antibody resulted in depletion of certain cell subsets rather than binding only was derived by staining peripheral blood and spleen cells from α-ASGM1–treated mice with a GAR second-step reagent that recognizes the α-ASGM1 heavy chain (data not shown). Table 2 summarizes the depletion data from the 3 treatment groups. As shown, α-ASGM1 not only depletes NK1.1$^+$ASGM1$^+$ [45] in vivo, but also depletes CD3$^+$ASGM1$^+$ and CD8$^+$ASGM1$^+$, but not Mac-1$^+$ASGM1$^+$, cells. Notably, mice conditioned with XRT alone demonstrated a significant relative increase in NK1.1$^+$ASGM1$^+$ cells, whereas the CD3$^+$ASGM1$^+$ and CD8$^+$ASGM1$^+$ cells were moderately to severely reduced. In mice that received the combination of XRT plus α-ASGM1 (the group with the lowest level of resistance), the NK1.1$^+$ASGM1$^+$ population was significantly depleted, as were the CD3$^+$ASGM1$^+$ and CD8$^+$ASGM1$^+$ cells.

CD8$^+$ Cells and Engraftment Barrier

To assess the contribution of the CD8$^+$ cells independent of NK cells to the engraftment barrier in this strain combination, HSC transplantations were performed in C57BL/6 recipients that lack expression of the CD8 molecule (B6.CD8α$^{-/-}$). Figure 3 shows that at the lower doses of HSCs, many more B6.CD8α$^{-/-}$ recipients were rescued as compared with wild-type mice. Ninety percent of B6.CD8α$^{-/-}$ mice that underwent transplantation with 6000 HSCs (P = .937 and .134), 70% that underwent transplan-

Table 2. *Flow Cytometric Analysis of B6.WT Splenocytes*

Treatment	% Live Cells	Lineage$^+$ASGM1$^+$ Splenocytes					
		NK1.1$^+$	CD3$^+$	CD4$^+$	CD8$^+$	Gr-1$^+$	Mac-1$^+$
None	80%	2.9%	9.4%	4.3%	3.0%	1.3%	3.4%
α-ASGM1	75%	0.2%	2.4%	2.7%	0.7%	0.7%	3.6%
XRT	18%	14.7%	1.9%	2.8%	1.4%	0.1%	0.7%
XRT + α-ASGM1	33%	0.9%	0.4%	2.2%	0.1%	0.1%	27.2%

Flow cytometric detection of double-stained splenocytes (lineage$^+$ASGM1$^+$) of different treatment groups. A 3-step FACS analysis was performed before and at day +7 after treatment (staining antibodies: unconjugated α-ASGM1, FITC-conjugated goat anti-rabbit, and phycoerythrin-conjugated lineage marker). The data shown are from a single representative animal in each treatment group and are expressed as percentages of > 20 000 recorded cells.

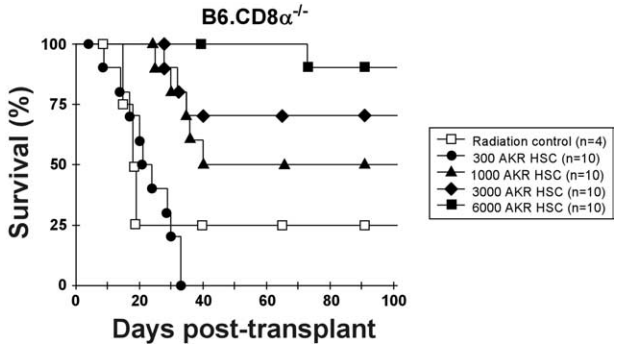

Figure 3. Transplantation of allogeneic HSCs into recipients lacking expression of the CD8 antigen. Titrated doses of MHC-mismatched AKR/J HSCs were injected into lethally irradiated CD8-deficient mice (B6.CD8$\alpha^{-/-}$). Shown is the survival of mice that underwent transplantation with 300, 1000, 3000, or 6000 HSCs compared with the survival of XRT controls (950 cGy of radiation). Results are pooled from 2 independent experiments.

tation with 3000 HSCs (P = .055 and .002), and 50% that underwent transplantation with 1000 HSCs (P = .011 and .014) survived longer than 100 days after transplantation. Reduction in the barrier was not as profound as that observed in mice that were treated with α-ASGM1, because unlike the α-ASGM1–treated group, in which 60% survival was observed at the 300-HSC dose, none of the B6.CD8$\alpha^{-/-}$ mice survived at the 300-HSC dose level. FACS staining of the peripheral blood and splenocytes of B6.CD8$\alpha^{-/-}$ mice before and 7 days after XRT revealed a marked percentage increase cells in NK cells after XRT, as measured by double staining for ASGM1$^+$ and NK1.1$^+$ (peripheral blood: 0.8% before versus 6.2% after XRT; splenocytes: 2.1% before versus 17.1% after XRT). Of note, similar to the chimerism data obtained from mice rescued with lower doses of HSCs in α-ASGM1–treated mice, a significant percentage of recipient B6.CD8$\alpha^{-/-}$ mice rescued with 1000 AKR HSCs were reconstituted by host-derived, but not donor-derived, hematopoiesis.

Effect of Elimination of Pathways of Cytotoxicity

Both NK and CD8$^+$ cells eliminate targets by common effector mechanisms that include granule-mediated (perforin and granzymes) and FasL-mediated killing. To delineate the relative importance of these cytotoxic pathways on HSC resistance in this strain combination, we performed transplantations into C57BL/6 mice that were defective for either perforin (B6.pfp$^{-/-}$) or FasL (B6.gld). Titrated numbers of AKR/J HSCs were infused into irradiated B6.pfp$^{-/-}$ or B6.gld recipients. Figure 4 shows that no discernible reduction in the barrier to engraftment was detectable in either immune-deficient mouse strain as compared with B6.WT controls. Results were obtained from 2 separate experiments, and P values are shown for the individual experiments. A total of 56% (P = .937 and .44) of perforin-deficient mice and 80% (P = .937 and .426) of FasL-defective mice that underwent transplantation with 6000 HSCs survived 100 days after transplantation. Survival at HSC doses <6000 was also comparable to that of B6.WT controls.

Elimination of Cytokines

In addition to the perforin and FasL cytotoxic pathways, NK and CD8$^+$ cells can kill or alter the function of target cells via production and secretion of immune-modulating cytokines. TNF-α and TGF-β have both been described as potentially responsible for allogeneic hematopoietic cell resistance [36,46]. We therefore examined the role of these cytokines, as well as the role of Th1- and Th2-associated cytokines, in engraftment resistance by using as recipients selected cytokine knockout mice. Graded numbers of AKR/J HSCs were infused into mice that were deficient in either TNF-α or TGF-β. Alternatively, mice deficient in either the Th1-polarizing cytokine interferon (IFN)–γ or the Th2 cytokines interleukin (IL)–4 or IL-10 served as recipients. Figure 5 dem-

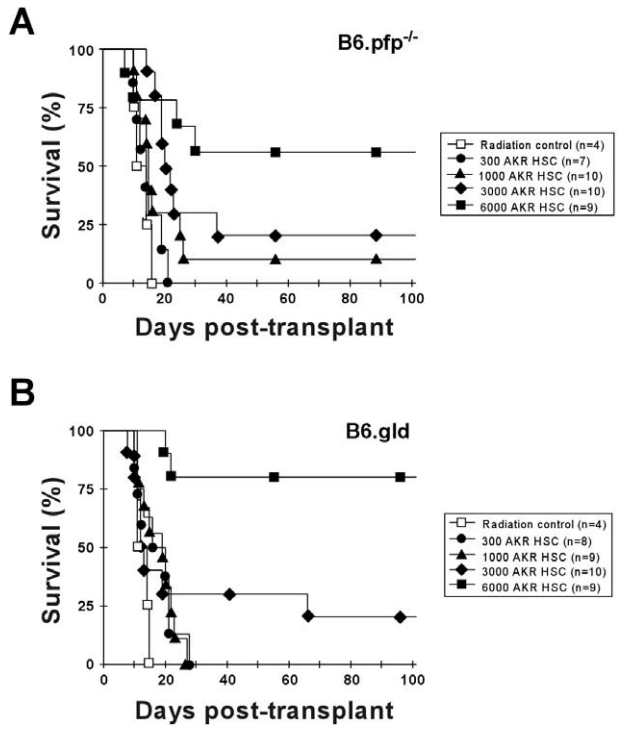

Figure 4. Transplantation of allogeneic HSCs into recipients with defects in the perforin or Fas pathways. Titrated doses of MHC-mismatched AKR/J HSCs were injected into lethally irradiated (A) perforin-deficient (B6.pfp$^{-/-}$) or (B) FasL-defective (B6.gld) recipients. Survival of mice that underwent transplantation with 300, 1000, 3000, or 6000 HSCs is compared with that of XRT controls (950 cGy of radiation). Results are pooled from 2 independent experiments.

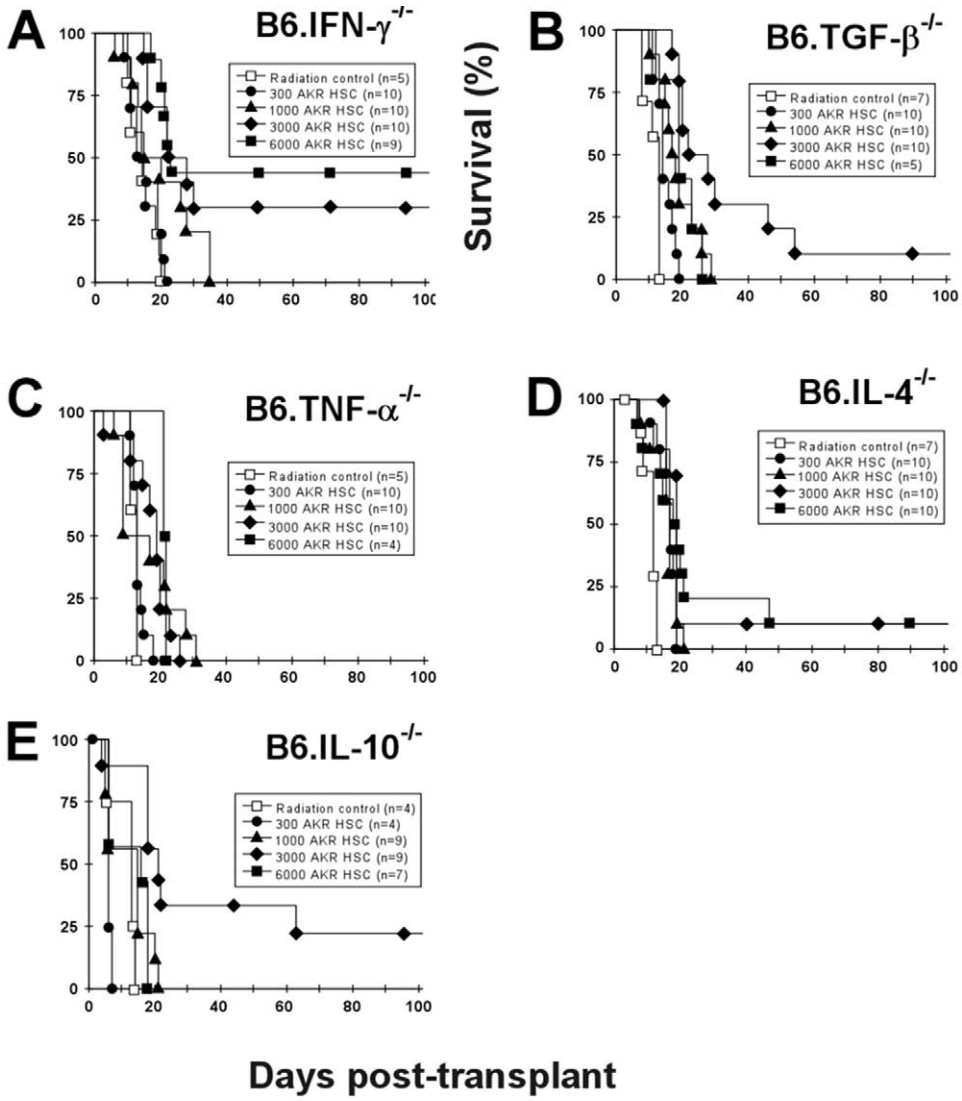

Figure 5. Transplantation of allogeneic HSCs into recipients defective in a single cytokine. Titrated doses of MHC-mismatched AKR/J HSCs were injected into lethally irradiated C57BL/6 mice defective for (A) IFN-γ, (B) TGF-β, (C) TNF-α, (D) IL-4, or (E) IL-10. Survival of mice that underwent transplantation with 300, 1000, 3000, or 6000 HSCs is compared with that of XRT controls (950 cGy of radiation). Results are pooled from 2 independent experiments for each treatment group.

onstrates that no reduction in the engraftment barrier was observed in any of the cytokine-deficient strains as compared with B6.WT mice. In fact, in many of the immune-deficient strains (TGF-$\beta^{-/-}$, TNF-$\alpha^{-/-}$, IL-$4^{-/-}$, and IL-$10^{-/-}$), worse survival was observed at the 6000-HSC dose compared with B6.WT recipients (TGF-$\beta^{-/-}$, $P = .007$; TNF-$\alpha^{-/-}$, $P = .004$; IL-$4^{-/-}$, $P = .032$ and .094; IL-$10^{-/-}$, $P = .008$ and .177). For the 2 cytokine knockout recipient groups, TGF-$\beta^{-/-}$ and IL-$10^{-/-}$, that did not demonstrate a strict dose-response relationship at the 3000- and 6000-HSC doses, these differences were not statistically significant ($P = .113$ and $P = .254$, respectively). Thus, we conclude that the absence of any 1 of these cytokines does not alter host resistance to allogeneic HSCs as measured in our model system.

Chimerism Analysis in Mice with Selected Immunologic Defects

Peripheral blood chimerism analysis was measured 6 weeks after transplantation in all surviving recipients from the treatment groups described previously. Table 1 and Table 3 summarize these chimerism data. Surviving mice were deemed donor chimeras if they had evidence of multilineage donor cells in the peripheral blood. Although chimeric mice had evidence of significant levels of persistent host T cells after transplantation, most demonstrated >60% donor T cells in the blood by 6 weeks. Estimates of non–T-cell chimerism were based on the percentage of donor $B220^+$ cells, which stain brightly for $H\text{-}2^k$ and which correlate with the levels of donor granulocytes and

Table 3. *Survival and Chimerism Analysis after Transplantation of Purified HSCs from AKR/J into B6 Immune-Defective Recipients*

Recipient	% Survival/% Donor Chimerism for HSC Dose (No. Mice Analyzed)			
	300 KTLS	1000 KTLS	3000 KTLS	6000 KTLS
B6.CD8α$^{-/-}$	0/0 (10)	50/20 (10)	70/100 (10)	90/100 (10)
B6.pfp$^{-/-}$	0/0 (7)	10/100 (10)	20/50 (10)	56/100 (9)
B6.gld	0/0 (8)	0/0 (9)	20/0 (10)	80/0 (9)
B6.IFN-γ$^{-/-}$	0/0 (10)	0/0 (10)	30/0 (10)	40/50 (9)
B6.TGF-β$^{-/-}$	0/0 (10)	0/0 (10)	10/100 (10)	0/0 (5)
B6.TNF-α$^{-/-}$	0/0 (10)	0/0 (10)	0/0 (10)	0/0 (4)
B6.IL-4$^{-/-}$	0/0 (10)	0/0 (10)	10/0 (10)	10/100 (10)
B6.IL-10$^{-/-}$	0/0 (4)	0/0 (9)	20/100 (9)	0/0 (5)

Shown are the percentage of recipients that survived for a given HSC dose and the percentage of these survivors that were chimeric. Mice were assessed for evidence of donor chimerism in the T- and B-cell and macrophage and granulocyte lineages at 6 weeks after HSC transplantation by flow cytometric analysis with donor class I H2^{k}-specific staining. Chimeric mice had > 60% donor-derived blood T cells. Non–T-cell chimerism was best quantitated by α-B220 staining, which was, on average, > 95% donor type. Nonchimeric recipients were < 5% donor type in all lineages. Data were pooled from 2 independent experiments. Recipients were prepared for transplantation with lethal radiation (950 cGy).

KTLS indicates *c-Kit*$^{+}$Thy-1^{lo}Lin$^{-/lo}$Sca-1^{+} HSCs.

macrophages [42]. Chimerism levels in these non–T-cell lineages were, on average, >95% by 6 weeks. Of note, survival did not always correlate with donor chimerism. As previously described, the most striking examples of differences between survival and donor chimerism occurred at the lower HSC doses in the α-ASGM1–treated mice (Table 1) and B6.CD8α$^{-/-}$ mice (Table 3). A lack of correlation between survival and chimerism was also noted in several of the immune-deficient mice at higher HSC doses, including B6.pfp$^{-/-}$, B6.gld, B6.IFN-γ$^{-/-}$, and B6.IL-4$^{-/-}$ recipients (Table 3). Of note, B6.gld recipients demonstrated resistance comparable to that of B6.WT mice (Tables 1 and 3); however, even at the highest HSC dose level, there was no evidence of donor-derived hematopoiesis. It therefore seems that in many of the experimental groups, persistent host hematopoietic elements survived the conditioning treatment and were responsible for long-term reconstitution of the blood.

DISCUSSION

Failure to engraft remains a potential life-threatening complication after AHCT. Clinical and animal studies have shown that manipulation of hematopoietic grafts can exacerbate this problem. In these studies, we attempted to dissect the mechanisms by which allogeneic HSCs are resisted by recipient immune cells. Prior reports by us and others have shown that mouse BM contains a non-HSC population that facilitates HSC engraftment [40,47]. Here, we transplanted purified *c-Kit*$^{+}$Thy-1^{lo}Lin$^{-/lo}$Sca-1^{+} HSCs. This allowed us to study resistance to engraftment in the absence of other confounding graft-facilitating populations.

The strain combination used in this study demonstrated high engraftment resistance to purified HSCs as compared with other allogeneic strains that we have tested, because even large doses of HSCs could not rescue 100% of lethally irradiated recipients. To establish the identity and role of immune effector cells in this model, HSC transplantations were performed into either mice that were treated with α-ASGM1 or CD8^{+} cells. α-ASGM1 depletes its target cells in vivo [48-51] and has been shown to block both NK cell and CTL activity after in vivo administration [43,44]. As expected, we found that the major population depleted by the antibody was also positive for NK cell markers. In addition, antibody treatment resulted in depletion of minor populations of CD3^{+}, CD4^{+}, and CD8^{+} cells that double-stain with α-ASGM1. It is interesting to note that the macrophage marker Mac-1 also stains ASGM1^{+} cells. However, in antibody plus XRT–treated mice, a prominent Mac-1^{+} population remained (Table 2), thus suggesting that this α-ASGM1^{+}Mac-1^{+} population does not contribute significantly to engraftment resistance. Transplantations into B6.CD8α$^{-/-}$ mice also showed a significant reduction in the engraftment barrier. This decrease was not as profound as in α-ASGM1–treated mice, in agreement with the observation that B6.CD8α$^{-/-}$ recipients retained significant levels of radioresistant ASGM1^{+}NK1.1^{+} cells. Thus, in the absence of facilitating cells, engraftment of purified HSCs across a strongly resistant allogeneic barrier can be achieved by eliminating or reducing radioresistant NK and/or CD8^{+} effector cell populations.

We then sought to study the mechanism(s) by which effector host populations mediate this allogeneic HSC engraftment barrier. Disruption of either of the 2 major cytotoxic pathways, the perforin-dependent or Fas-FasL interaction, did not alter HSC resistance. These results were consistent with prior reports in different transplantation models. Aguila and Weissman [35] studied transplantation of purified al-

logeneic HSCs in transgenic mice deficient in NK and T-cell cytotoxicity due to cell suicide triggered by expression of granzyme A. Graubert et al. [37] studied Hh resistance in granzyme B–deficient mice, and Baker et al. [36] tested allogeneic BM resistance in perforin knockout mice and FasL-deficient recipients. In all 3 independent reports, it was found that impairment of killing via granule exocytosis did not measurably reduce the hematopoietic cell resistance. Furthermore, the study by Baker et al. [36] and our studies showed that FasL-deficient mice also maintained strong engraftment barriers. More recently, Komatsu et al. [39] generated double-deficient (perforin plus FasL) and triple-deficient (perforin plus FasL plus TNF receptor) mice that served as recipients for hematopoietic cell transplants across minor histocompatibility barriers. Neither the double nor triple cytotoxic–deficient mice showed a diminished capacity to resist BM allografts as compared with wild-type recipients. Of note, studies by Bennett et al. [52] compared the need for perforin- and Fas-dependent pathways in acute rejection of BM by NK cells in 2 different strains (129 and C57BL/6 mice). Lack of perforin was noted to decrease the ability of 129 mice, but not C57BL/6 mice, to reject incompatible BM. However, this dependence on perforin was highly influenced by the environmental housing conditions. Thus, data from several independent laboratories showed that these 2 primary mechanisms of cellular-mediated cytotoxicity likely do not represent the principal way that hematopoietic grafts are eliminated.

An alternative mechanism to perforin or Fas-FasL pathways is that secreted cytokines play the primary role in resisting allogeneic hematopoietic cells. Here we used as recipients several knockout mice that were defective in production of defined cytokines and found that no single cytokine defect reduced the allograft barrier. Because of prior studies suggesting that TNF-α, TGF-β, or IFN-γ has a regulatory role in hematopoiesis [53] and/or can mediate killing of target cells, the transplantations performed into these cytokine-deficient mice were of particular interest. TNF-α and IFN-γ have been shown to induce Fas antigen expression on the surface of human HSCs, thus increasing their susceptibility to killing by anti-Fas antibodies [54,55]. TNF-α also has direct cytotoxic actions, and it was suggested that TNF-α might regulate hematopoiesis by inhibiting the growth of early hematopoietic cells [56-58]. Nonetheless, neither the TNF-α– nor IFN-γ–deficient mice showed a reduction in the engraftment barrier as compared with wild-type mice. In fact, in both of these knockout mouse strains, resistance seemed more severe, with reduced survival and reduced chimerism, respectively. The proposed roles of TGF-β have been conflicting. It is thought that TGF-β can mediate veto effects that result in the deletion of alloreactive CTLs [36,59]. Alternatively, TGF-β may act to decrease NK cell potency by inhibiting the expression of FasL on NK cells. The latter hypothesis suggests that TGF-β–deficient mice would demonstrate increased—not reduced—resistance to engraftment. In our hands, TGF-β–deficient mice demonstrated increased resistance to HSCs compared with wild-type recipients, which, taken together with the TNF-α or IFN-γ data, supports a role for these cytokines in the reduction of barrier activity rather than its augmentation. Mice with selected defects in either Th1 or Th2 polarizing cytokines similarly had either no reduction or increased resistance to engraftment. It is intriguing that recipients lacking IL-4, IL-10, TGF-β, and TNF-α demonstrated increased resistance compared with wild-type mice. These observations suggest that selected cytokine interactions may promote rather than deter engraftment. We are currently carrying out experiments treating knockout mice with α-ASGM1 to determine whether high levels of resistance remain in these cytokine-deficient mice under conditions known to optimally reduce the engraftment barrier.

One notable finding was that survival after myeloablative XRT did not directly correlate with chimerism outcome in several of the experimental groups (Tables 1 and 3). The most obvious discordance was observed at the lower HSC dose levels in the α-ASGM1–treated or the CD8 knockout recipients, which demonstrated superior survival as compared with wild-type mice. We interpret these data to mean that a reduction in immune-mediated resistance allows donor HSCs to provide transient rescue of blood-forming capacity during a critical window of time after XRT, but that surviving endogenous HSCs abolish the donor cells and take over the hematopoietic niches. It should be emphasized that our measures of engraftment (ie, survival and chimerism) allowed evaluation of long-term reconstitution and differed from assays such as splenic 125-iododeoxyuridine uptake, which is a short-term assay often used as a surrogate for long-term engraftment [19,60]. That rare host HSCs can survive myeloablation and eventually repopulate wild-type hosts is supported by a recent study demonstrating that lethally irradiated mice were rescued with progenitor cells restricted to the megakaryocyte/erythrocyte lineage [61]. Such progenitor cells did not have the capacity to self-renew, and the radioprotected mice had 100% host-derived hematopoiesis beyond 30 days after transplantation. Large numbers of donor HSCs apparently (except in the case of gld recipients) prevent the re-emergence of host hematopoiesis, and the importance of stem cell dose to engraftment outcome has been already described for syngeneic [62] and allogeneic [40,63] transplantations. However, it remains to be clarified in the allogeneic setting whether, in fact, donor HSCs compete for hematopoietic niches or give rise to pop-

ulations that suppress host HSCs. The observation that even high doses of HSCs in FasL-defective gld mice did not result in donor chimerism implies that disruption of the Fas/FasL pathway provides protection of their HSCs from the ablative effects of XRT and/or provides an as yet undefined advantage over donor HSCs. Regardless, our studies reinforce the concept relevant to clinical transplantation that the HSC dose contained in an allogeneic hematopoietic graft is critical for achieving sustained engraftment.

Understanding how HSCs are recognized and resisted is a complex biological question. From our studies and others modeling hematopoietic resistance in mice, it is evident that different cell populations contribute to resistance and that their role(s) are influenced by multiple factors, including genetic mismatch, graft content, recipient preparation, and even environmental components [52]. The fact that we did not observe a significant decrease in HSC resistance by elimination of a single cytotoxic mechanism further supports the idea that several synergistic elements control HSC engraftment. Proper homeostatic regulation of blood production is fundamentally important to the survival of an organism, and we believe that many of the elements that control allogeneic HSC resistance are the same as those that regulate physiologic hematopoiesis. Thus, unraveling how transplanted HSCs are targeted and suppressed will undoubtedly contribute to our understanding of the control of normal hematopoiesis and to the understanding of pathologic states in which endogenous hematopoiesis is suppressed, such as aplastic anemia and hypoplastic myelodysplastic syndrome.

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